

## Inhibition of *Streptococcus mutans* Biofilm Formation by *Streptococcus salivarius* FruA<sup>▽</sup>

Ayako Ogawa,<sup>1,3</sup> Soichi Furukawa,<sup>2</sup> Shuhei Fujita,<sup>2</sup> Jiro Mitobe,<sup>3</sup> Taketo Kawai,<sup>3</sup> Naoki Narisawa,<sup>3</sup> Tsuyoshi Sekizuka,<sup>4</sup> Makoto Kuroda,<sup>4</sup> Kuniyasu Ochiai,<sup>5</sup> Hirokazu Ogiwara,<sup>2</sup> Saori Kosono,<sup>6</sup> Saori Yoneda,<sup>3</sup> Haruo Watanabe,<sup>3</sup> Yasushi Morinaga,<sup>2</sup> Hiroshi Uematsu,<sup>1</sup> and Hidenobu Senpuku<sup>3\*</sup>

Department of Gerodontology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan<sup>1</sup>; Department of Food Science and Technology, College of Bioresource Sciences, Nihon University, Kanagawa, Japan<sup>2</sup>; Department of Bacteriology<sup>3</sup> and Laboratory of Bacterial Genomics,<sup>4</sup> Pathogen Genomics Center, National Institute of Infectious Diseases, Tokyo, Japan; Department of Bacteriology, Nihon University of Dentistry, Tokyo, Japan<sup>5</sup>; and Environmental Molecular Biology Laboratory, RIKEN, Saitama, Japan<sup>6</sup>

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The oral microbial flora consists of many beneficial species of bacteria that are associated with a healthy condition and control the progression of oral disease. Cooperative interactions between oral streptococci and the pathogens play important roles in the development of dental biofilms in the oral cavity. To determine the roles of oral streptococci in multispecies biofilm development and the effects of the streptococci in biofilm formation, the active substances inhibiting *Streptococcus mutans* biofilm formation were purified from *Streptococcus salivarius* ATCC 9759 and HT9R culture supernatants using ion exchange and gel filtration chromatography. Matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometry analysis was performed, and the results were compared to databases. The *S. salivarius* HT9R genome sequence was determined and used to identify candidate proteins for inhibition. The candidates inhibiting biofilms were identified as *S. salivarius* fructosyltransferase (FTF) and exo-beta-D-fructosidase (FruA). The activity of the inhibitors was elevated in the presence of sucrose, and the inhibitory effects were dependent on the sucrose concentration in the biofilm formation assay medium. Purified and commercial FruA from *Aspergillus niger* (31.6% identity and 59.6% similarity to the amino acid sequence of FruA from *S. salivarius* HT9R) completely inhibited *S. mutans* GS-5 biofilm formation on saliva-coated polystyrene and hydroxyapatite surfaces. Inhibition was induced by decreasing polysaccharide production, which is dependent on sucrose digestion rather than fructan digestion. The data indicate that *S. salivarius* produces large quantities of FruA and that FruA alone may play an important role in multispecies microbial interactions for sucrose-dependent biofilm formation in the oral cavity.

Dental biofilms are produced by bacterial communities with large biodiversity (>700 bacterial species) (1, 28, 39) and high densities (~10<sup>11</sup> cell/g of wet weight) (20). During the first 24 h of colonization, oral streptococci compose 60% to 90% of the supragingival plaque biomass (32, 37). Various streptococci form the oral microflora and have multiple functions in cooperation and in competition with mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) and other streptococci. These multiple interactions are based on the biology of the streptococci as well as on the physical effects of previous attachments, and the colonization regulates biofilm formation using multiple species of mutans streptococci and other commensal streptococci in the oral cavity ecosystem.

Mutans streptococci are biofilm-forming bacteria and are considered to be the primary etiologic agents of human dental caries (19, 30). They possess a variety of abilities to colonize tooth surfaces and under certain conditions are present in large quantities in cariogenic biofilms and also

form biofilms with other organisms, including other streptococci and bacteria (13, 30, 36). Mutans streptococci produce glucosyltransferase (GTF) enzymes that synthesize glucan from the glucose moiety of sucrose that causes the cariogenicity of the dental pathogens. Although many studies have described the mechanisms of streptococcal adhesion (17, 24, 37, 41) and coaggregation among oral bacteria (25, 38), the subsequent process of bacterial accumulation, proliferation, and biofilm formation leading to functional heterogeneous species in the organized sessile community is poorly understood, especially in the presence of sucrose that induces cariogenicity. However, it is likely that the cooperative interactions between mutans streptococci and other oral streptococci play important roles in the development of dental biofilm and caries in the oral cavity (27, 48, 50, 53).

*Streptococcus salivarius* is a typical representative of the commensal microflora residents in the oral cavity and is a major constituent of the biofilm colonizing the buccal epithelium, tongue, and dorsal epithelium (6). This microorganism comprises the majority of the total cultivable flora on the soft tissues of the mouth and possesses a number of important biological activities in lactose uptake and urease production that are thought to contribute to the stability of the oral community (15, 44). *S. salivarius* metabolic products may regulate

\* Corresponding author. Mailing address: Department of Bacteriology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Phone: 81 3 5285 1111. Fax: 81 3 5285 1163. E-mail: hsenpuku@nih.go.jp.

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oral biofilm formation over a considerable distance to integrate the entire oral cavity (18, 44). When *S. salivarius* was cocultured with *S. mutans* GS-5, *S. salivarius* inhibited *S. mutans* biofilm formation (48). A substance(s) in the culture supernatant from *S. salivarius* inhibited the activities of competence-stimulating peptide (CSP) for biofilm formation on polystyrene plates and competency in *S. mutans* (48). *S. salivarius* colonizes the surfaces of the oral cavity and is believed to remain a numerically important member of this ecosystem throughout life.

Fructosyltransferase (FTF) is an enzyme that converts sucrose to extracellular homopolymers of fructose, the fructans. Streptococci have at least two types of FTF activity which differ in the structure of the products, inulin ( $\beta 2 \rightarrow 1$  fructan) or levan ( $\beta 2 \rightarrow 6$  fructan). *S. salivarius* produces FTF that converts sucrose to levan (21). Bacteria that produce fructans also produce enzymes that degrade these polymers (10). Conversely, strains of *S. mutans* and other streptococci generally produce extracellular enzymes that hydrolyze the two primary types of polymers of D-fructose produced from sucrose using the FTF from oral bacteria (8, 16, 51). This enzyme, the product of the *fruA* gene, is an exo- $\beta$ -D-fructosidase that releases fructose from  $\beta(2,6)$ - and  $\beta(2,1)$ -linked fructans and also cleaves fructose from sucrose and raffinose (9, 11). FTF and FruA were demonstrated to aid in the development of dental caries using various mutant strains of *S. mutans* (12, 23, 35, 42).

Here, we identified the inhibitors produced by *S. salivarius* that interfere with *S. mutans* biofilm formation. We purified the proteins, performed a proteome analysis, compared the purified protein to commercial enzymes, and observed the interactions between *S. mutans* and products from *S. salivarius* using a quantitative measurement of biofilm formation on the surface of saliva-coated 96-well polystyrene plates and hydroxyapatite supplemented with sucrose. The protein analysis showed that the substance causing inhibition of the biofilm was identified as a fructanase from the *S. salivarius* culture supernatant. We show that *S. salivarius* FruA may modulate sucrose-dependent colonization of *S. mutans* on oral cavity surfaces.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** We used *S. mutans* GS-5 and *S. salivarius* HT9R, JCM5707, and ATCC 9759. All bacteria were grown in an aerobic atmosphere of 5% CO<sub>2</sub>, 75% N<sub>2</sub>, and 20% O<sub>2</sub> (GasPac CO<sub>2</sub>; Becton/Dickinson, Sparks, MD) in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) at 37°C prior to inoculation into 96-well microtiter plates. The bacterial growth was measured as the absorbance at 600 nm at 0, 1, 2, 3, 4, 5, 6, 7, 9, and 22 h after inoculation of *S. salivarius* HT9R, ATCC 9759, or JCM5707 into tryptic soy broth (TSB; Difco Laboratories) without dextrose and with 0.25% sucrose. *S. mutans* GS-5 was cultivated with 0, 0.9, 1.9, 3.8 and 7.5 units/ml FruA in a fructanase mixture for 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 h in TSB without dextrose and with 0.25% sucrose. The bacterial growth was measured as the absorbance level at 600 nm after ultrasonic dispersion (60-W power output) for 10 s.

**Biofilm formation assay.** Biofilm formation using each strain was assayed using a method described previously (48). To evaluate biofilm formation using a dual-species culture of oral streptococci, 20  $\mu$ l of one bacterial cell suspension ( $4.0 \times 10^4$  CFU) (or phosphate-buffered saline [PBS] as a control) was mixed with 20  $\mu$ l of another cell suspension ( $4.0 \times 10^4$  CFU) in 160  $\mu$ l of TSB without dextrose and supplemented with 0.25% sucrose or 3% sucrose in 96-well (flat-bottom) microtiter plates (Sumitomo Bakelite, Tokyo, Japan). To evaluate the effects of the samples from *S. salivarius* and commercial fructanase, 20  $\mu$ l of a *S. mutans* GS-5 suspension ( $4.0 \times 10^4$  CFU) was mixed with 20  $\mu$ l of samples from *S. salivarius* and a fructanase mixture (Megazyme, Wicklow, Ireland) that includes exo-inulinase (FruA)-to-endo-inulinase unit ratios of 10:1 or less of fruc-

tanase activity, exo-inulinase (Megazyme) and endo-inulinase (Megazyme) from *Aspergillus niger*, and 160  $\mu$ l of TSB with 0.25% sucrose. The biofilm formation assay was performed at 37°C for 10 h or 16 h using 5% CO<sub>2</sub> in an aerobic atmosphere. After the plates were incubated at 37°C for 10 or 16 h, the liquid medium was removed, and the wells were rinsed twice with sterile distilled water (DW). The plates were then air dried and stained with 0.25% safranin-0.5% ethanol-H<sub>2</sub>O for 15 min. After plates were stained, they were rinsed with DW to remove the excess dye and then air dried. The biofilm mass was dissolved with 70% ethanol and measured using a microplate reader (Thermo Bioanalysis Japan, Tokyo, Japan). Quantification of the stained biofilm was performed by measuring the absorbance at 492 nm.

**Substrate hydrolysis activity.** To assay for fructan or sucrose hydrolysis, soluble fructan (inulin; Nonomoist, Bioland, Chungnam, South Korea) or sucrose (Wako Chemical, Tokyo, Japan) was prepared, respectively. The fructanase was assayed for activity by measuring the appearance of fructose or glucose released from the substrate: 1% inulin-DW or 1% sucrose-DW and TSB with 0.25% dextran (Sigma). Activity was quantitatively expressed as a concentration (units mg<sup>-1</sup> of protein). Fructose or glucose was utilized as the standard for the reaction. Monosaccharide concentration was defined using the method of Bradford (7) with a commercially available kit (Fructanase Assay Kit [Sigma-Aldrich] or Glucose CII-test [Wako Chemical]).

**Preparation of inhibitors from *S. salivarius*.** BHI extract medium was prepared using dialysis of 37 g of BHI powder/100 ml of DW in a cellulose dialysis membrane (passage molecular weight, 14,000; Viskase Companies, Inc., Darien, IL) in 1 liter of ultrapure DW. *S. salivarius* ATCC 9759 or HT9R was inoculated into the BHI extract medium with or without 0.25% sucrose and incubated at 37°C overnight. Culture supernatant samples were precipitated using gradual addition to 55% ammonium sulfate at 4°C. The precipitates were collected by centrifugation at 10,000  $\times$  g, resuspended in 20 mM Tris buffer (pH 7.4), and dialyzed against Tris buffer at 4°C. The sample was then concentrated using an ultrafiltration centrifugal filter with a cutoff of >10 kDa (Amicon Ultra, Millipore, Billerica, MA). The supernatant sample was applied to a DEAE-Sephacel column (GE Healthcare Bioscience, Buckinghamshire, United Kingdom) pre-equilibrated with the same buffer. After the column was washed extensively, 7-ml fractions were collected using an elution gradient from 0.15 M to 0.25 M NaCl in 20 mM Tris-HCl buffer at a flow rate of 0.2 ml/min and monitored at UV 280-nm absorbance. The fractions showing absorbance were assayed for suppression of biofilm formation. The active fractions were pooled and concentrated using ultrafiltration with a >10-kDa cutoff. The samples were applied to a Superdex 200 column (20 mm by 750 mm; GE Healthcare Bioscience) equilibrated with 20 mM Tris-HCl buffer, pH 7.4. The column was washed with the buffer at a flow rate of 0.3 ml/min, and these fractionated samples were monitored at UV 280 nm.

**MALDI-TOF mass spectrometry and peptide mass fingerprinting.** After SDS-PAGE, the silver-stained protein bands were excised from the gels, and in-gel digestion with trypsin was performed as described previously (26). Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectra were acquired using a Bruker Ultraflex mass spectrometer (Bruker Daltonics Inc., Billerica, MA). The peptide masses were compared to the MSDB database using the Mascot search engine ([http://www.matrixscience.com/cgi/search\\_form.pl?FORMVER=2&SEARCH=PMF](http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF)). Search parameters were defined with a mass accuracy of  $\pm 0.5$  Da and one missing cleavage by trypsin, carbamidomethylation of cysteine, and oxidation of methionine.

**Measurement of polysaccharide.** Cell suspensions of *S. mutans* GS-5 were cultivated with 1.25 or 6.0 units of fructanase/ml at 37°C for 16 h using 5% CO<sub>2</sub> in an aerobic atmosphere in TSB with 0.25% sucrose using six-well culture plates. The liquid medium was removed, and the wells were rinsed twice with sterile distilled water (dH<sub>2</sub>O). To observe polysaccharide in the biofilm, biofilm cells on the six-well plates and planktonic cells were treated with 2 mg/ml dextran-Alexa Fluor 647 (10,000 molecular weight [MW], anionic, and fixable; Invitrogen, Carlsbad, CA) in PBS (pH 8.0) for 30 min at room temperature. After samples were washed with PBS three times, the biofilms or planktonic cells in PBS were poured on glass slides covered with a coverglass. Alexa Fluor-labeled polysaccharide was observed using a fluorescence microscope (BX50; Olympus, Tokyo, Japan).

**Statistics.** Comparison of biofilm formation levels among various cultures of single and dual species were performed using analysis of variance (ANOVA). A *P* value of 0.05 or less was considered to be statistically significant.

**Nucleotide sequence accession number.** The sequence fragment of *S. salivarius* HT9R determined in this study was deposited in the DDBJ database under accession number DRA000072.

## RESULTS

**Biofilm formation in dual-species culture.** In a previous report, we found that biofilm formation decreased in dual-species cultures using *S. salivarius* HT9R and *S. mutans* GS-5 in comparison to single-culture biofilm formation with *S. mutans* GS-5 (48). We proposed the possibility that *S. salivarius* produced factors to inhibit *S. mutans* biofilm formation. Here, we examined an *S. salivarius* HT9R, JCM5707, or ATCC 9759 strain that was cultivated with *S. mutans* strain MT8148, GS-5, or ATCC 25175 in dual-species culture. *S. salivarius* HT9R, JCM5707, and ATCC 9759 showed significantly decreased *S. mutans* biofilm production, but the inhibition level was less with *S. salivarius* JCM5707 than with *S. salivarius* HT9R or ATCC 9759 (data not shown). The two primary inhibitory strains also inhibited biofilm formation in the dual-species culture with other streptococci (data not shown). Therefore, we observed a variation in the biofilm-decreasing effects using the *S. salivarius* strains. The data suggest that *S. salivarius* HT9R and ATCC 9759 may have strong inhibitors and may produce inhibiting factors for *S. mutans* sucrose-dependent biofilm formation.

***S. salivarius* growth and fructanase production.** To characterize the *S. salivarius* strains, the growth of the strains was measured in TSB without dextrose and with 0.25% sucrose, the same medium that was used in the biofilm formation assay. Growth of *S. salivarius* HT9R was faster than that of other strains in the lag phase and similar to that of others in the log phase; growth of *S. salivarius* ATCC 9759 reached the same level as that measured for *S. salivarius* HT9R in the stationary phase (data not shown). However, growth of *S. salivarius* JCM5707 did not reach the same level as that of other strains measured in the stationary phase. We measured the fructanase activity in culture supernatants of the *S. salivarius* strains after 6 h of incubation in BHI medium at 37°C. The activity was significantly higher in *S. salivarius* HT9R and ATCC 9759 than in *S. salivarius* JCM5707. Therefore, the variation among *S. salivarius* strains in the effects inhibiting biofilm formation may be dependent on the production of fructanase or the growth level of individual strains.

**Characterization of biofilm inhibitors from *S. salivarius*.** To purify the inhibiting substances from *S. salivarius*, precipitations of protein samples in the supernatant after culture in BHI extract medium alone or in BHI extract medium with 0.25% sucrose were performed using ammonium sulfate salting. Active substances such as FTF and FruA in *S. salivarius* are more highly secreted into the medium in the presence of sucrose (2, 14, 34). Therefore, BHI extract medium including sucrose was used for cultures, and the supernatant was precipitated to collect the protein samples. The ammonia sulfate precipitate (ASP) samples from *S. salivarius* ATCC 9759 and HT9R were dissolved in PBS, applied at various concentrations, and incubated in fresh cell suspensions of *S. mutans* GS-5 in TSB with 0.25% sucrose for 16 h at 37°C in saliva-coated 96-well microtiter plates. The effects of the ASPs on biofilm formation were assessed in a dose-dependent application, and biofilm formation was compared to the activities of ASPs in BHI medium with and without sucrose. ASPs from *S. salivarius* ATCC 9759 in medium with and without sucrose and ASP from HT9R in the medium with sucrose inhibited biofilm formation dose de-

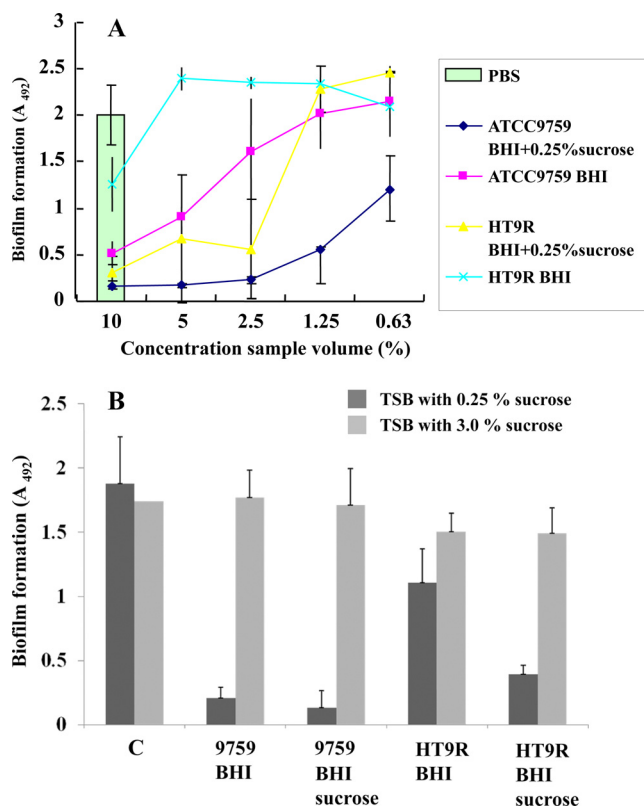


FIG. 1. Inhibition of biofilm using *S. salivarius* ATCC 9759 cultures in BHI broth with or without 0.25% sucrose. (A) The graphs show quantification of biofilms formed after 16 h in cultures of *S. mutans* GS-5 using 0.63%, 1.25%, 2.5%, or 10% ASP sample volumes from *S. salivarius* ATCC 9759 or HT9R in the culture medium with or without 0.25% sucrose in 96-well microtiter plates. (B) The biofilm formation assays were performed in *S. mutans* GS-5 suspensions with 10% ASP samples in medium with 0.25% or 3.0% sucrose. As a control, PBS was substituted in the biofilm formation assay. The results are expressed as the means  $\pm$  standard deviations of absorbance obtained in triplicate assays. Representative data from three independent experiments are presented, and similar results were obtained from each experiment.

pendently in comparison to the control (addition of PBS) (Fig. 1A). ASP from *S. salivarius* HT9R in the medium without sucrose inhibited biofilm formation at a 10% concentration only. The inhibition level was significantly higher in the ATCC 9759 and HT9R strains when BHI broth plus 0.25% sucrose was used than when BHI broth was used alone. From the data, the inhibitory factors were highly induced in the medium with sucrose. We next tested whether increasing the substrate, sucrose, affected the inhibition activity by ASP in *S. mutans* GS-5 biofilm formation. Biofilm formation of *S. mutans* is primarily produced using GTF, and the biofilm was inhibited by the addition of 10% ASP to the medium including 0.25% sucrose. However, in a medium with 3% sucrose, no inhibition was observed, and biofilm formation remained at the control level (Fig. 1B). Therefore, the inhibition was sucrose concentration dependent.

**Purification and identification of biofilm inhibitors.** To identify the inhibiting proteins in the ASP from *S. salivarius*, we applied the ASP to a DEAE-Sephacel column (ion exchange chromatography), and, further, the concentrated active frac-



tions were applied to a Superdex 200 column (gel filtration chromatography). Each fractionated sample from each chromatography was screened for inhibition activity with *S. mutans* GS-5 using the biofilm assay. The specific activity (arbitrary standard units) for *S. salivarius* ATCC 9757 increased from 4.51 U mg<sup>-1</sup> in the crude supernatant to 15.76 U mg<sup>-1</sup> and 85.66 U mg<sup>-1</sup> in the purified fractions after DEAE-Sephacel and Superdex 200 chromatography, respectively. After gel filtration using Superdex 200, fractionated samples were tested using the biofilm formation assay. Some fractions (numbers 10, 11, and 12) showed similar inhibiting activities as the ASP, whereas other fractions (numbers 8, 9, and 13) did not show inhibition activity (Fig. 2A). Then, we characterized the fractions using SDS-PAGE and silver stained them where the protein bands were cut out; we analyzed the fractions using MALDI-TOF mass spectrometry and compared them to potential genome sequences identified in *S. salivarius* HT9R. We determined the sequence of a short fragment of the genome of *S. salivarius* HT9R at the Genome Center of the National Institute of Infectious Diseases. Because the genomic DNA sequence of *S. salivarius* was not published, we required this genetic data to find candidate proteins for inhibition. These sequences are available in the DDBJ database (accession number DRA000072). Two proteins (~115 kDa and 80 kDa) were identified as FruA and FTF (Fig. 2B). A small band between FruA and FTF was a nonsecreting protein, the 50S ribosomal protein. We purified and identified the inhibiting proteins from the ASP sample from *S. salivarius* HT9R culture supernatant using the same method as for ATCC 9757 and found FTF and a glucan binding protein at 95 kDa and 50 kDa, respectively.

**Biofilm inhibition using FruA.** FruA is an enzyme that converts fructan to fructose by hydrolyzation. It is also known to hydrolyze sucrose. FruA may be important for *S. salivarius* metabolism and colonization, allowing the organism to survive in the severe environment of the oral cavity. FruA is also an important substance for *S. mutans* virulence (12). The genes that encode FruA have been compared in various streptococcus and fungus strains. To compare the conserved regions of FruA, the identity and similarity in amino acid sequence were determined. The amino acid sequence of FruA from *S. salivarius* HT9R showed 66.2% identity and 86.7% or 86.6% similarity to the *S. mutans* clinical isolate NN2025 or the laboratory strain UA159, respectively, and 95.5% identity and 97.7% similarity to *S. salivarius* SK126, for which the genome sequence was recently determined. There was some similarity to *Streptococcus gordonii*, with lower identity, and similarity with lower identity to *A. niger* SCB, the commercial source of FruA. FruA was identical in *S. salivarius* HT9R and SK126. To confirm the effects of FruA activity on inhibition of *S. mutans* biofilm formation, the fructanase mixture from *A. niger* that has significant FruA activity was used. The fructanase mixture contained exo-inulinase and endo-inulinase (ratio at 10 to <1, respectively). Various concentrations of the fructanase mixture were added to the growth medium of *S. mutans* in TSB with 0.25% sucrose. As a result, fructanase did not inhibit growth; however, fructanase inhibited biofilm formation of *S. mutans* on the saliva-coated 96-well microtiter plate surface in a dose-dependent manner (Fig. 3A). In contrast, fructanase activity increased in a dose-dependent manner (Fig. 3A). There was a negative correlation between biofilm inhibition and fructanase

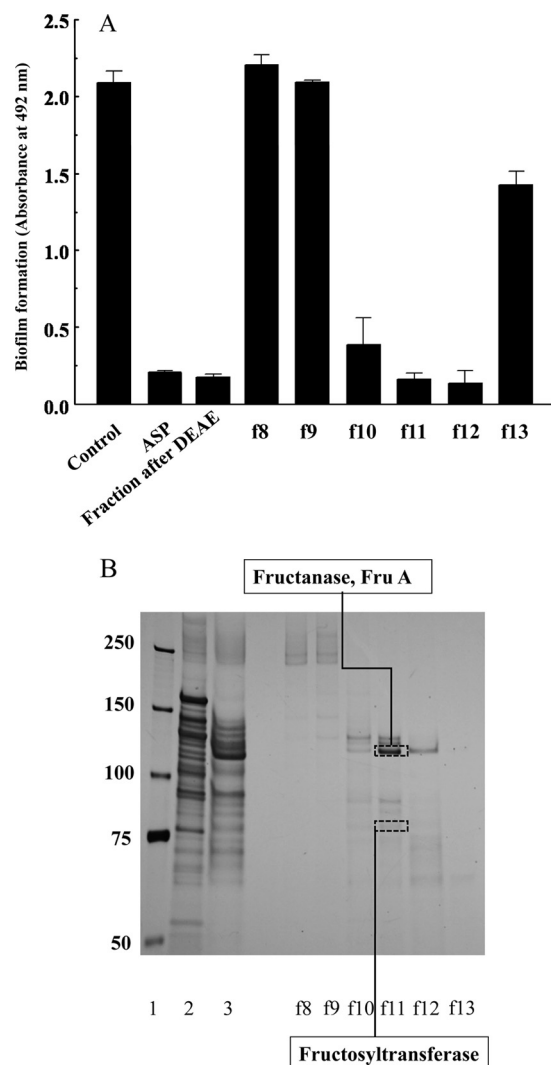


FIG. 2. Biofilm formation assays and SDS-PAGE using samples from *S. salivarius* ATCC 9759 after DEAE ion exchange chromatography. (A) *S. salivarius* ATCC 9757 samples (f8 to f13) fractionated using gel filtration with DEAE ion exchange chromatography were tested using the biofilm formation assay using *S. mutans* GS-5. The results are expressed as the means  $\pm$  standard deviations of absorbance at 492 nm obtained from three wells/assay. Representative data from three independent experiments are presented, and similar results were obtained in each experiment. Control, PBS addition. (B) SDS-PAGE of samples fractionated from gel filtration chromatography where protein bands were identified using silver stain. Representative data from three independent experiments are presented, and similar results were obtained in each experiment. Lane 1, marker; lane 2, ASP, lane 3, sample after DEAE ion exchange chromatography (active fraction); f8 and f9, inactive fractions; f10 to f12, active fractions; f13, inactive fraction after gel filtration chromatography. Dotted squares indicate two candidate proteins for biofilm inhibition.

activity using a gradual increase in FruA concentration. The negative correlation was also observed in biofilm formation of *S. sobrinus* (data not shown). The inhibition activity by FruA was also confirmed in safranin-stained biofilm on saliva-coated hydroxyapatite disks with FruA at 1.25 units/ml and 6 units/ml, the minimum and low concentrations, respectively, for biofilm inhibition on 96-well microtiter plates

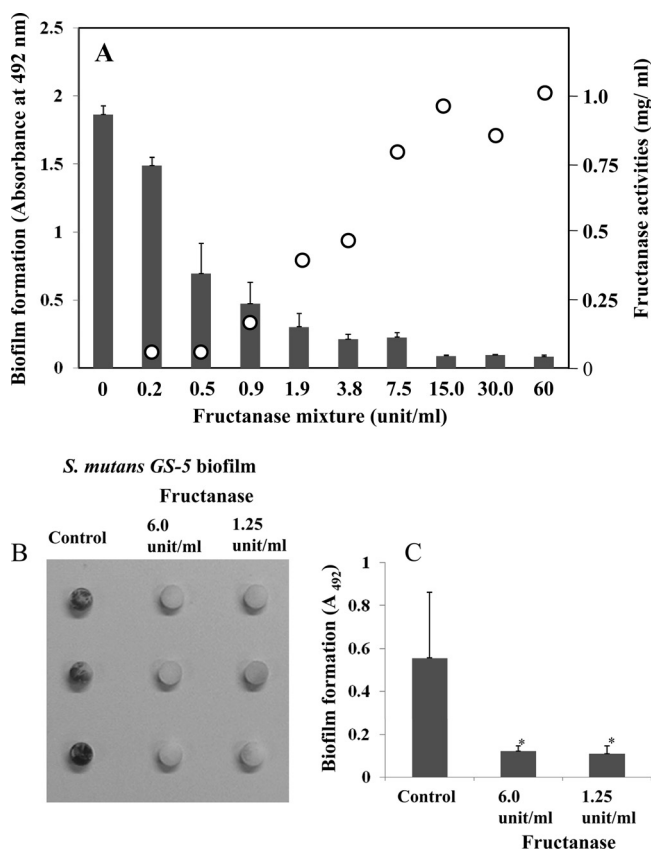


FIG. 3. Biofilm formation of *S. mutans* treated with FruA on saliva-coated polystyrene and hydroxyapatite surfaces. (A) Various units of FruA in the fructanase mixture were added and incubated with an *S. mutans* cell suspension in saliva-coated 96-well microtiter plates for 16 h in TSB with 0.25% sucrose and 1% inulin-DW. Fructanase activities in the cell suspension were measured as the fructose concentration after 2 h of incubation. (B) Effects of FruA in the fructanase mixture on biofilm formation of *S. mutans* GS-5 were analyzed on saliva-coated hydroxyapatite disks. Three hydroxyapatite disks were used: control (PBS), 1.25 unit/ml, and 6.0 unit/ml FruA. A biofilm photograph was taken as representative data from one of the three experiments. (C) Quantitative analyses were also performed for biofilm formation on hydroxyapatite. The results are expressed as the means  $\pm$  standard deviations of the absorbance obtained in triplicate assays in panel B. Representative data of three independent experiments are presented, and similar results were obtained in each experiment. The asterisks denote significantly different biofilm formation comparing the FruA treatment and control ( $P < 0.05$ ).

(Fig. 3B). When the biofilms were dissolved with 70% ethanol, we found that FruA significantly reduced the biofilm (Fig. 3C). Exo-inulinase alone or endo-inulinase alone was also added into the biofilm formation assay. Exo-inulinase alone completely inhibited the biofilm, whereas the endo-inulinase did not inhibit the biofilm (data not shown). Therefore, the activities of the fructanase mixture were dependent on the exo-inulinase. To characterize the thermal properties of the fructanase, it was heated to various temperatures and used in the biofilm formation assay. Significant inhibition was maintained after it was heated to 62°C for 10 min, whereas inhibition was lost at >64°C for 10 min.

**Inhibition by FruA in polysaccharide formation.** We do not believe that the conversion of fructan to fructose is the primary

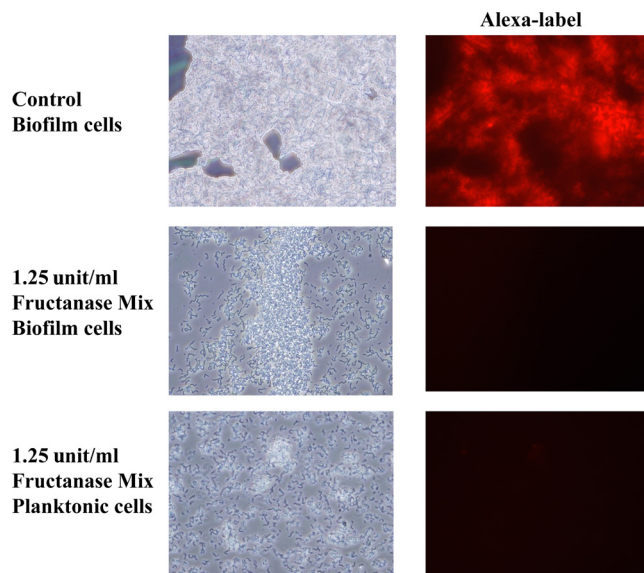


FIG. 4. Polysaccharide analysis using a biofilm formation assay of *S. mutans* GS-5 treated with 1.25 units/ml FruA mixture. The biofilm and planktonic cells and the polysaccharide labeled with Alexa Fluor were observed in the left column with visible light and in the right column with fluorescence. Representative data of three independent experiments are presented, and similar results were obtained in each experiment.

mechanism of biofilm inhibition by FruA because inulin is not employed as a principal ingredient in *S. mutans* biofilm formation in the presence of sucrose. To examine the effects of FruA on glucan formation (Fig. 4) in the formation of the biofilm, dextran-Alexa Fluor 647 was used to label the polysaccharide, and fluorescence was observed using microscopy in the control biofilm (Fig. 4, top right photograph). However, biofilm and planktonic cells treated with 1.25 units/ml FruA did not show fluorescence (Fig. 4, bottom middle photograph).

**Sucrose digestion by FruA.** FruA has the ability to digest the substrate, sucrose, in the medium. Therefore, sucrose was used as a substrate to observe digestion by FruA. FruA (30 U/ml) was added as an excess concentration in a 1% sucrose solution in DW, and after incubation for 2 h at 37°C, the glucose concentration was measured. Around 600 mg/dl glucose was observed in the sample treated with 30 units/ml fructanase mixture and 10 units/ml exo-inulinase (high concentrations for biofilm inhibition) (Fig. 5A). Glucose production was significantly observed at 1.25 unit/ml FruA (data not shown). ASP samples used in the experiment shown Fig. 1 from *S. salivarius* HT9R and ATCC 9759 cultured in medium with and without sucrose showed glucose concentrations of >350 mg/dl. The active fractions for *S. mutans* biofilm inhibition after the DEAE-Sephacel ion exchange chromatography shown in Fig. 2A yielded glucose at >350 mg/dl, and these active fractions (numbers 10, 11, and 12) after the Superdex 200 gel filtration shown in Fig. 2A yielded significant glucose concentrations. Thus, the purified FruA sample had the same ability as the commercial FruA for sucrose digestion.

To study the inhibition time course of fructanase, 30 units/ml FruA was added at various time points to an *S. mutans* GS-5 suspension, and the biofilm formation level was investigated

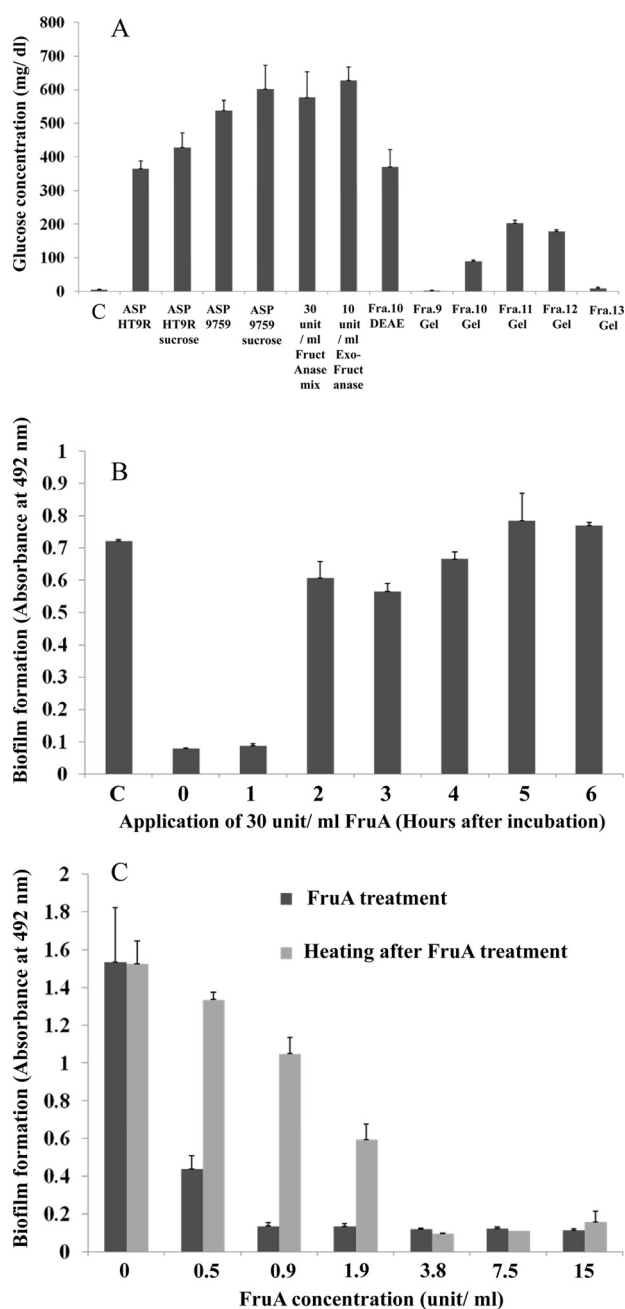


FIG. 5. Sucrose digestion using treatment with FruA. (A) Glucose production was analyzed in the sucrose solutions treated with various samples: ASPs from *S. salivarius* HT9R and ATCC 9759 with or without sucrose; 30 units/ml FruA mixture; 10 units/ml exo-inulinase alone; fractions 9, 10, 11, 12, and 13 after gel filtration chromatography; and the same samples as shown in Fig. 3. (B) Thirty units/ml FruA was added at 0, 1, 2, 3, 4, 5, and 6 h after incubation of *S. mutans* GS-5 in TSB with 0.25% sucrose on a saliva-coated 96-well microtiter plate. The biofilm formation assay was performed for 10 h. (C) TSB with 0.25% sucrose was treated with 0, 0.5, 0.9, 1.9, 3.8, 7.5, or 15.0 units/ml FruA at 37°C for 1 h before the addition of *S. mutans* GS-5. The treated medium was heated at 70°C for 10 min and used as the medium for the biofilm formation assay on the saliva-coated 96-well microtiter plates. A comparison of biofilm formation levels in medium treated with heated and unheated FruA is shown. The results are expressed as the means  $\pm$  standard deviations of absorbance obtained in triplicate assays. Representative data of three independent experiments are presented, and similar results were obtained in each experiment.

until the end of log phase (10 h) because the differences were defined at the incubation time. Applications of FruA at 0 and 1 h after culture start, but not later, showed significant inhibition (Fig. 5B). This indicates that FruA affects early formation of the biofilm. FruA may digest sucrose completely in the medium before glucan production by GTF, which is secreted after 1 h of culture of *S. mutans*. To determine the effects using FruA in sucrose digestion, the biofilm assay medium TSB with 0.25% sucrose was treated with various units/ml of FruA and heated to the inactivation temperature, 70°C. This was performed because the remaining activity of FruA toward *S. mutans* after pretreatment may disturb the analysis of the inhibition effects resulting from sucrose digestion. The pretreatment by FruA at more than 3.8 units/ml in both heated and non-heated samples completely inhibited *S. mutans* biofilm formation (Fig. 5C). The complete inhibition effect in the unheated sample was maintained when FruA was diluted to 0.9 units/ml from 15 units/ml. However, the inhibition effects with FruA at less than 1.9 units/ml in the heated samples were restored to various levels. This indicated that the FruA activity remained after the treatment with FruA in TSB with 0.25% sucrose; therefore, biofilm inhibition remained in the unheated sample.

## DISCUSSION

Two *S. salivarius* strains consistently inhibited biofilm formation in our assay using dual-species cultures with *S. mutans* GS-5 (48) and inhibited biofilms as well with other strains and streptococci. *S. salivarius* is a non-biofilm-forming bacterium and does not directly communicate with biofilm bacteria on the tooth surface; however, it inhibits biofilm formation by streptococci in the *in vitro* assay. Previously, *S. salivarius* was found to produce urease enzymes that are thought to contribute to the stability of oral communities (15, 44). *S. salivarius* is the primary bacteria species inhabiting the oral cavity and is distributed throughout the cavity by saliva. Here, we show that FruA produced by *S. salivarius* inhibited *S. mutans* biofilm formation completely in the *in vitro* assay supplemented with sucrose. This was also supported by the inhibition activities of commercial fructanase from *A. niger*. The purified samples from *S. salivarius* and commercial FruA induced upregulation of glucose concentrations in the sucrose solution, which is a substrate for glucan synthesis by GTF (Fig. 5A). FruA inhibited biofilm formation early in the biofilm formation assay (Fig. 5B). Further, the biofilm did not develop in the culture medium containing sucrose pretreated with FruA before the biofilm assay (Fig. 5C). This suggests that FruA digests sucrose before the production of glucan by GTF, which is secreted after one or more hours of culture of *S. mutans* in the medium, and that this glucose after the digestion was not employed for synthesis of glucan. Fructan is not an essential component in streptococcal biofilm formation. Therefore, we propose that the FruA mechanism is the cleaving of sucrose into glucose and fructose rather than inulin digestion.

This study shows that FruA has various identities and similarities among microorganisms. We show that commercial FruA from *A. niger* with a low identity but similar to other streptococcal FruA inhibited *S. mutans* biofilm formation completely and showed the same fructanase activity as the FruA purified from the *S. salivarius* supernatant. Further, FruA also



inhibited *S. sobrinus* biofilm formation. This demonstrates indirectly that fructanase activity may nonspecifically be as important for biofilm development under conditions with supplemented sucrose. FruA produced not only by *S. salivarius* but also by other streptococci is everywhere in the oral cavity. This suggests that FruA may play an essential role in the development of oral biofilm formation by commensal bacteria and may regulate microbial pathogenicity in the oral cavity.

FruA converts fructan to a fructose; however, this is not the cause for the inhibition of *S. mutans* biofilm formation. *S. mutans* lacks a complete tricarboxylic acid (TCA) cycle in its respiratory chain; thus, the organism depends entirely on glycolysis to generate sufficient energy for growth (3, 4, 49), and, therefore, FTF and FruA are needed for the carbohydrate utilization required for growth (55). The synthesis and subsequent degradation of fructans from sucrose generate energy to enhance growth to increase the cariogenic potential of the organisms. FTF was also identified in the inhibiting samples from *S. salivarius* ATCC 9759 and HT9R. FTF converts sucrose to fructan; therefore, 0.25% sucrose in the medium of the biofilm formation assay is consumed by FTF, and the GTF-dependent biofilm formation of *S. mutans* may have decreased because of a reduction in the required sucrose concentration. However, the function may be to store carbohydrate after the conversion using its bacterial metabolism. This allows *S. mutans* to shunt a readily diffusible substance into a high-molecular-weight polymer that can be accessed over an extended period of time. Further, we suggest that this causes increased amounts of carbohydrate to be converted to acids, increasing the duration of exposure of tooth mineral to a low pH (12, 13).

Previous reports used isogenic FruA mutants in a programmed rat caries model to show that FruA is a virulence determinant contributing to the progression of dental caries (12). This presents a discrepancy in the role of FruA for pathogenic activity of *S. mutans* between our data and previous reports. Possibly, in our *in vitro* study, exogenous FruA was used in the biofilm formation assay, and, therefore, the enzyme responded quickly to a substrate such as sucrose. In contrast, the isogenic FruA mutant that was used in previous *in vivo* reports may not respond quickly and needs time for cell growth, signaling, and production (12). Therefore, the roles of FruA may be variable and dependent on experimental, environmental, and growth conditions for various streptococci. Another possibility is that the sufficient concentration of sucrose (0.25%) declined after FruA addition, reducing biofilm formation, whereas excess concentrations of sucrose were used in the feed for the rats in the previous *in vivo* assay. Sucrose concentration is the key for pathogenic activity of *S. mutans*. However, we observed that biofilm formation was not inhibited by addition of samples from *S. salivarius* where increased concentrations of sucrose from 0.25% to 3% were used (Fig. 1B). However, the continuous ingestion of food containing excess amounts of sucrose (56%) is not unusual. The role of FruA may depend also on a balance of sucrose concentrations and the homeostatic bacterial metabolism in various environments during biofilm formation and the appearance of pathogenic activity. Recently, a report suggested that some aspects of diet-induced changes in the microbiota composition may be predetermined based on the intrinsic capacity of an individual species to use the sub-

strates being consumed by the host (46). The investigators speculate that diets enriched in different polysaccharides could result in microbiotas of very different species composition. Therefore, substrates such as sucrose should be used carefully to study *S. mutans* pathology to try to simulate concentration levels similar to natural levels in either *in vitro* or *in vivo* assays.

The glucan binding protein (Gbp) was also identified in the inhibiting substances from *S. salivarius* HT9R. The biological significance of the Gbps has not been defined; however, studies suggest that these proteins influence virulence and play a role in maintaining biofilm architecture by linking bacteria to extracellular molecules of the glucan (5, 43). The Gbps consist of GbpA, GbpB, GbpC, and GbpD. GbpA, GbpC, and GbpD are secreted, and only GbpA and GbpD are released. The extracellular GbpA and GbpD contribute to the scaffolding that allows *S. mutans* to build onto the biofilm outward from the substratum (31). The precise functions of the Gbp have yet to be clearly defined; however, previous reports suggest that *S. mutans* Gbps positively affects biofilm formation. Therefore, we considered that Gbp is not an inhibiting factor in the ASP from *S. salivarius* for *S. mutans* biofilm formation.

Recently, our laboratory reported that inhibitor proteins from *S. salivarius* HT9R induced inactivation of the competence-stimulating peptide (CSP) and inhibited CSP-dependent bacteriocin production and biofilm formation by *S. mutans* (48). *Streptococcus gordonii* also produces an inhibitor that degrades the CSP and inhibits a bacteriocin produced by *S. mutans* GS-5 (53). CSP encoded by *comC* induces competence and antimicrobial activity and is one of the key factors in streptococcal biofilm formation (40). Therefore, products from *S. salivarius* including FruA may inhibit CSP-dependent competency, bacteriocin production, and biofilm formation. The issues remaining are further studies concerning the interactions between *S. mutans* and *S. salivarius*.

A variety of nonspecific  $\beta$ -D-fructofuranosidases are found in bacteria, yeast, and fungi. For example, inulinases and levanases are capable of hydrolyzing inulin; levan and sucrose have been isolated from *Bacillus subtilis* (29), *Actinomyces viscosus* ATCC 15987 (33), *S. mutans* (9), *Saccharomyces fragilis* (45), and *Chrysosporium pannorum* (54). Enzymes active for inulin and sucrose but not levan are found in filamentous fungi among the  $\beta$ -fructofuranosidases (I to III) from *A. niger* (52) and *C. pannorum* (54). However, levanases which are specific for levan have been isolated from *S. salivarius* KTA-19 (47) and *Actinomyces viscosus* ATCC 19246 (2). Here, we show that the exo-inulinase from *S. salivarius* ATCC 9759 has similar functions to the exo-inulinase from *A. niger* in addition to being a levanase. This enzyme function may regulate the biofilm inhibition activity for *S. mutans* and other streptococci in *in vitro* assays supplemented with sucrose. Therefore, the fructanase from the streptococci may have multiple properties depending on the species, genospecies, and strain. The differences in substrate preferences may be important in the oral cavity, where there are probably multiple types of fructans available as a result of the variable compositions of oral biofilms at different sites in the mouth.

In conclusion, here we demonstrate that FruA plays a central role in inhibition of biofilm formation by multiple species including *S. salivarius*. However, other factors from *S. salivarius*

may influence biofilm formation, in addition to the influence of FruA and CSP activities. FruA from other streptococci was also involved in streptococcus biofilm formation. Further investigations are needed to evaluate whether the purified recombinant proteins of FruA from various streptococci and mutants of FruA and other components from various streptococci support the multispecies communication mechanisms of the streptococci.

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